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The C-terminal pentapeptide of LHRH is a dominant B cell epitope with antigenic and biological function

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Abstract

Luteinizing hormone releasing hormone (LHRH) has been intensively studied as a target for the control of fertility and hormone dependent cancers. In most studies a decapeptide, EHWSYGLRPG, which is identical to the native LHRH sequence, has been used. In this study we investigated whether short sequences of LHRH could retain immunogenic and antigenic properties and be employed in a vaccine preparation. Our results show that the C-terminal five-residue peptide ($LHRH^{6-10}$) of LHRH was able to inhibit the binding of anti-$LHRH^{1-10}$ antisera to $LHRH^{1-10}$ in an inhibition ELISA. A totally synthetic peptide vaccine incorporating $LHRH^{6-10}$ also elicited a strong anti-LHRH antibody response and prevented mice from becoming pregnant in fertility trials. The primary immune response elicited by a peptide vaccine based on $LHRH^{1-10}$ could be boosted with $LHRH^{6-10}$. Finally, an antigen system comprising of biotinylated $LHRH^{6-10}$ bound to streptavidin-coated plates was capable of discriminating between anti-LHRH antibodies present in fertile and non-fertile mice. This study demonstrates that $LHRH^{6-10}$ retains immunogenic and antigenic properties and also discerns antibody specificities associated with reproductive competence.

Keywords: Immunocontraceptive vaccine; Antibody; Antigenicity; Epitope; Peptide vaccine

1. Introduction

Luteinizing hormone releasing hormone (LHRH) is secreted by the hypothalamus and initiates a cascade of endocrine events in all mammals that leads to reproductive competency. Immunisation against LHRH has been proven to be an effective method for blocking this hypothalamic pituitary axis (Fraser and Baker, 1978; Fraser et al., 1974; Talwar et al., 1992) and as consequence vaccines incorporating LHRH have been intensively studied for the control of fertility and hormone dependent cancers (Naz et al., 2005; Talwar, 1999). In most studies the immunogen consists of the LHRH decapeptide, EHWSYGLRPG. Within the native sequence of LHRH the N-terminal residue is pyroglutamic acid, a cyclized analogue of glutamic acid, and the C-terminus is in the amide ($CONH_2$) form. Traditionally the peptide sequence is chemically conjugated to a protein carrier (Beekman et al., 1997, 1999, 2001; Ladd et al., 1990; Miller et al., 1997), incorporated into a recombinant immunogen (Jinshu et al., 2005; Talwar et al., 2004) or incorporated into a peptide vaccine containing $CD4^+$ T cell epitopes for induction of T cell help (Ghosh and Jackson, 1999; Ghosh et al., 2001; Meloen et al., 1994; Oonk et al., 1998; Sad et al., 1992; Zeng et al., 2002). Unlike carrier proteins, the use of synthetic T helper (T_H) cell epitopes has the potential advantage of by-passing carrier-induced epitope-specific suppression (Sad et al., 1992).

Our laboratory has been engaged in the preparation of totally synthetic contraceptive vaccines based on LHRH. We have shown that a totally synthetic peptide vaccine containing LHRH as the B cell epitope and a T_H cell epitope elicits a potent antibody response against LHRH in mice and that these mice are rendered infertile (Ghosh and Jackson, 1999; Zeng et al., 2002, 2005). We have used both full length LHRH and a homolog in which the N-terminal pyroglutamic acid residue is removed. In both cases the antibody responses induced were similar (Ghosh and Jackson, 1999; Zeng et al., 2001, 2002, 2005). An early report (Singh et al., 1985) indicated that antisera obtained following immunisation with an LHRH-tetanus toxoid conjugate

Abbreviations: LHRH, luteinizing hormone releasing hormone; Pam2Cys, *S*-[2,3-bis(palmitoyloxy)-propyl]-cysteine; T_{HFlu}, T helper cell epitope from the light chain (HA2) of influenza virus; T_{Hmob}, a T helper cell epitope from the canine distemper virus fusion protein

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were (i) able to inhibit follicular development and ovulation and (ii) reacted with full length LHRH but were devoid of reactivity with the peptide sequences SYG ($LHRH^{1-6}$), LRPG ($LHRH^{7-10}$) and with the free acid form of LHRH where the C-terminal glycine was in the carboxylate and not the amide form. On the other hand it has been reported that inoculation with LHRH-derived peptides in which His-2 and Trp-3 have been deleted are effective at inducing LHRH neutralising antibodies and causing immunocastration (Rigby et al., 1988).

These reports combined with our own results prompted us to investigate which part of the sequence of LHRH is the dominant B cell epitope and whether short sequences derived from LHRH could retain their immunogenic and antigenic properties when incorporated into peptide vaccines.

2. Materials and methods

2.1. Reagents

Unless otherwise stated chemicals were of analytical grade or its equivalent. Dichloromethane (DCM), *N,N'*-dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA), O'benzotriazole-*N,N,N',N'*-tetra methyl-uronium-hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIPEA) and diisopropylcarbodiimide (DICI) were obtained from Auspep Pty Ltd (Melbourne, Australia) and Fluka (Buchs, Switzerland). Phenol and triisopropylsilane (TIPS) were from Aldrich (Milwaukee, WI) and trinitrobenzylsulphonic acid (TNBSA) from Fluka (Buchs, Switzerland); 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) was obtained from Sigma. Fmoc amino acids were obtained from Auspep (Melbourne, Australia) or Merck Australia (Kilsyth, Australia).

2.2. Peptide synthesis and purification

A schematic of the peptides and lipopeptides used in this study is shown in Fig. 1. To prepare the LHRH peptide vaccines two different T helper cell epitopes were employed: a peptide from the light chain (HA2) of influenza virus hemagglutinin which has the sequence GALNNRFQIKGVELKS (Jackson et al., 1995; Zeng et al., 2001), or a T helper cell epitope, which was identified from the canine distemper virus fusion protein and has the sequence KLIPNASLIENCTKAEL (Ghosh et al., 2001).

Peptides and lipopeptides were synthesised in house using Fmoc chemistry throughout; details of the synthetic procedures used are to be found elsewhere (Zeng et al., 2005). The lipid moiety used to increase the immunogenicity of the peptide-vaccine was dipalmitoyl *S*-glyceryl cysteine (Pam2Cys). The characteristics of this self-adjuvanting group in the context of immunocontraceptive vaccines have been described by us previously (Jackson et al., 2004; Zeng et al., 2002). Peptides were cleaved from the resin and side chain protecting groups simultaneously removed by treatment with Reagent B (88% TFA containing 5% phenol, 5% water and 2% triisopropylsilane).

Analytical HPLC was carried out using a Vydac C4 column (4.6 mm × 300 mm) installed in a Waters HPLC system. Chromatogram were developed at a flow rate of 1 ml/min using

Peptide sequence	Abbreviation
^{1}EHWSYGLRPG^{10}	$LHRH^{1-10}$
^{1}EHWYS^{5}	$LHRH^{1-5}$
^{2}HWSYG^{6}	$LHRH^{2-6}$
^{3}WSYGL^{7}	$LHRH^{3-7}$
^{4}SYGLR^{8}	$LHRH^{4-8}$
^{5}YGLRP^{9}	$LHRH^{5-9}$
^{6}GLRP^{9}	$LHRH^{6-9}$
^{6}GLRPG^{10}	$LHRH^{6-10}$
^{7}LRPG^{10}	$LHRH^{7-10}$

GALNNRFQIKGVELKS helper epitope $T_{H\,flu}$

KLIPNASLIENCTKAEL helper epitope $T_{H\,morb}$

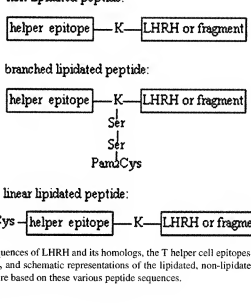

Fig. 1. Sequences of LHRH and its homologs, the T helper cell epitopes $T_{H\,Flu}$ and $T_{H\,morb}$ and schematic representations of the lipidated, non-lipidated vaccines that are based on these various peptide sequences.

0.1% TFA in H_2O and 0.1% TFA in acetonitrile as the limit buffer. Purification of peptide constructs was carried out using a semipreparative Vydac C4 column (10 mm × 300 mm) installed in an FPLC (Pharmacia Pty. Ltd) system using the same solvent system as for analytical HPLC except that a flow rate of 2 ml/min was used. All peptides eluted as single major peaks by analytical HPLC. Mass analysis was carried out using an Agilent 1100 Series Capillary LC system in-line with an Agilent 1100 Series LC/MSD ion-trap mass spectrometer. The mass spectrometer was operated with electrospray ionisation configured in the positive ion mode. All purified peptides and lipopeptides had the expected mass.

2.3. Inoculation protocols

Groups of five female BALB/c mice, 6–8 weeks old, were inoculated subcutaneously on day 0 with a dose of peptide

vaccine containing 20 nmole of LHRH formulated in complete Freund's adjuvant (CFA). The mice received a second, similar dose of vaccine on day 28 in incomplete Freund adjuvant (IFA). Miee were bled 4 weeks (i.e. 28 days) after the primary inoculation and 2 weeks following the secondary inoculation i.e. on day 42. The lipidated versions of peptide vaccines, in which the lipid group Pam2Cys was coupled to the epsilon group of the intervening lysine residue placed between the two epitopes, were dissolved in saline and administered using similar inoculation regimes exeept that no extraneous adjuvant was used. Sera for analysis of antibody content were prepared from blood obtained on days 28 and 42.

2.4. Enzyme-linked immunosorbent assays (ELISAs)

These assays were carried out on serum samples as described previously (Zeng et al., 2005) using either LHRH or the C-terminal pentapeptide ($LHRH^{6-10}$) of LHRH, or their biotinylated forms as the coating antigen. In the case where stretavidin was used to increase the binding of biotinylated LHRH and $LHRH^{6-10}$, streptavidin was used first to coat the plates at 5 μg/ml in PBSN3 overnight and then the biotinylated peptide at a concentration of 5 μg/ml in PBSN3 was added and incubated overnight. Serial dilutions of antisera were prepared and incubated overnight with antigen-coated plates. Antibody titres were expressed as the reciprocal of the highest dilution of serum achieving an optical density of 0.2; this value represents approximately five times the optical absorbance observed in the absence of anti-peptide antibody. Antibody binding is sometimes expressed directly as the absorbance at 405 nm.

2.5. Inhibition assays

Inhibition ELISA assays were performed essentially as described elsewhere (Sadler et al., 1999; Tang et al., 1988). Briefly, antisera were obtained at day 42 from mice that had received two doses (days 0 and 28) of the non-lipidated form of peptide vaccine incorporating $LHRH^{1-10}$ and the T helper cell epitope $T_H Flu$. The vaccine was administered in the presenee of Freund's adjuvant on day 0 and in incomplete Freund's adjuvant on day 28. Serial dilutions of the inhibitor peptides, $LHRH^{1-10}$, $LHRH^{1-5}$, $LHRH^{2-6}$, $LHRH^{3-7}$, $LHRH^{4-8}$, $LHRH^{5-9}$, $LHRH^{6-10}$, $LHRH^{7-10}$ and $LHRH^{6-9}$ were prepared in saline and mixed with a constant amount of antisera that, in the absence of inhibitor, gave less than maximum binding. The mixtures were incubated for 2 h at room temperature before being added to $LHRH^{1-10}$-coated wells. After overnight incubation, bound antibody was detected as described above. The results are expressed as the percentage of the antibody bound in the absenee of inhibitor.

2.6. Fertility studies in mice

Fertility studies were carried out as described previously (Zeng et al., 2001). Briefly female mice were examined for their ability to drop litters following inoculation with peptide immunogens and exposure to untreated male mice. A male mouse was introduced to either two or three female miec 2 weeks after females had received the second dose of vaccine. Males were rotated between each group of females in order to expose each female to every male. Males and females were kept togethter for a total of 3 weeks at the end of whieh time males were removed and the females kept under observation. A group of female mice inoculated with saline emulsified in CFA was used as a control.

2.7. Statistical analysis

The statistical analyses of the antibody data was carried out using two-tailed Student's *t*-test calculated using Prism software and each of the resultant *p* values for a particular comparison is shown in the appropriate text or in figure legend.

3. Results

3.1. Inhibition of the binding of anti-LHRH antibodies to LHRH by peptide fragments

In this experiment we determined which part of the LHRH sequence was able to inhibit the binding of anti-LHRH antibodies raised against full length LHRH. Anti-LHRH antisera were obtained from mice inoculated with two doses of the peptide vaccine GALNNRFQIKGVELKS-$LHRH^{1-10}$ in the presence of CFA on days 0 and 28. The sequence GALNNRFQIKGVELKS is a T helper cell epitope identified from the light chain (HA2) of influenza virus hemagglutinin (Jackson et al., 1995; Zeng et al., 2001). Anti-LHRH antisera were prepared from blood obtained on day 42. A panel of six pentapeptides and two tetrapeptides derived from the LHRH sequence (Fig. 1) were used in the inhibition assays. The five pentapeptides used cover the complete sequence of LHRH and are overlapping by four residues. The two tetrapeptides cover only the C-terminal five residues and are overlapping by three residues. The anti-LHRH antisera were incubated with each of the peptides for 2 h before adding to wells coated with $LHRH^{1-10}$.

The results (Fig. 2) show that, of the peptides examined, the C-terminal pentapeptide $LHRH^{6-10}$ with sequence GLRPG was the most effective inhibitor and at a dose of 83 nmoles there is no difference between the inhibitor activities of $LHRH^{1-10}$ and $LHRH^{6-10}$. At lower concentrations the inhibitor activity of $LHRH^{6-10}$ was approximately 20% less efficient than $LHRH^{1-10}$. Of the two tetrapeptides: $LHRH^{7-10}$ (sequenee LRPG) and $LHRH^{6-9}$ (sequence GLRP), only the C-terminal tetrapeptide $LHRH^{7-10}$ showed modest inhibition ranging from 12 to 35% (Fig. 2). All other peptides showed an inhibition efficiency of less than 15%.

3.2. Comparison of the immunogenicity of the peptide vaccines based on $LHRH^{1-10}$, $LHRH^{6-10}$, or $LHRH^{7-10}$

The results of the inhibition ELISAs indicated that the C-terminal 4 (LRPG) or 5 (GLRPG) residues of LHRH were able to inhibit the binding of anti-LHRH antibody sera to $LHRH^{1-10}$. We therefore determined if these peptides were able to elicit

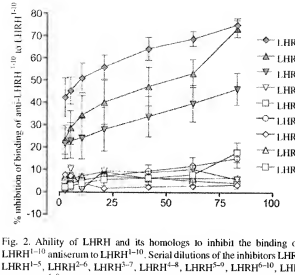

Fig. 2. Ability of LHRH and its homologs to inhibit the binding of anti-$LHRH^{1-10}$ antiserum to $LHRH^{1-10}$. Serial dilutions of the inhibitors $LHRH^{1-10}$, $LHRH^{1-5}$, $LHRH^{2-6}$, $LHRH^{2-7}$, $LHRH^{1-8}$, $LHRH^{1-9}$, $LHRH^{6-10}$, $LHRH^{7-10}$ and $LHRH^{6-9}$ were mixed with a constant amount of antiserum and held for 2 h at room temperature before being added to $LHRH^{1-10}$-coated wells and the amount of antibody bound subsequently determined by ELISA. The results are expressed as the percentage of antibody bound in the absence of inhibitor.

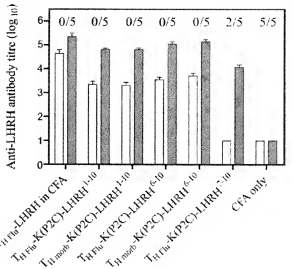

Fig. 3. The immunnogenicity of lipopeptide vaccines incorporating $LHRH^{1-10}$, $LHRH^{7-10}$ and $LHRH^{6-10}$ and $T_{H\,Flu}$ or $T_{H\,morb}$ as the T helper cell epitope. The anti-LHRH antibody titres were measured by ELISA using $LHRH^{1-10}$ as the coating antigen. The mean titre obtained from groups of five mice are shown with an error bar representing ±1 S.D. The open bars represent antibody titres obtained in the primary response and the filled bars represent the antibody titres obtained in the secondary response. The numbers at the top of the bars refer to the number of mice that dropped litters during the course of a fertility trial that was carried out 2 weeks following the second dose of vaccine.

anti-LHRH antibody responses and whether or not the antibodies elicited were functional. Five lipopeptides were made; three containing GALNNRFQIKGVELKS ($T_{H\,Flu}$) as the T helper cell epitope and either $LHRH^{1-10}$, $LHRH^{6-10}$ or $LHRH^{7-10}$ as the B cell epitope and the other two containing KLIPNASLIENCTKAEL ($T_{H\,morb}$) as the T helper cell epitope and either $LHRH^{1-10}$ or $LHRH^{6-10}$ as the B cell epitope. $T_{H\,morb}$ is a T helper cell epitope identified from canine distemper virus fusion protein (Ghosh et al., 2001) and is active both in BALB/c mice (Zeng et al., 2005) and dogs (Ghosh et al., 2001). The lipid group dipalmitoyl *S*-glyceryl cysteine (Pam2Cys) was attached to the epsilon group of an intervening lysine inserted between the two epitopes with two serine residues as a spacer (Zeng et al., 2002).

Each of the five lipidated peptides were administered subcutaneously into five BALB/c mice in saline and as a positive control a non-lipidated version of peptide vaccine containing $LHRH^{1-10}$ was administered subcutaneously in the presence of complete Freund's adjuvant. Mice received two doses of peptide vaccine on days 0 and 28 and were bled on days 28 and 42 and the anti-LHRH antibody responses measured by ELISA using $LHRH^{1-10}$ as antigen. The results (Fig. 3) showed (i) that two doses of lipopeptides containing $LHRH^{6-10}$ and administered in saline elicited as strong an anti-LHRH antibody response as that obtained when lipopeptide based on $LHRH^{1-10}$ administered in saline or non-lipidated peptide based on $LHRH^{1-10}$ and administered in CFA were used ($p > 0.05$) (ii) that when the C-terminal tetrapeptide $LHRH^{7-10}$-based vaccine was inoculated into mice, no significant anti-LHRH antibody response was detected following the first dose but a strong anti-LHRH response was obtained following the second dose of vaccine and (iii) no difference ($p > 0.05$) was observed in the antibody response elicited by using either $T_{H\,Flu}$ or $T_{H\,mob}$.

A fertility trial was also carried out to test the biological efficacy of the vaccines. Two weeks after the second dose of vaccines, male mice were introduced to the females and allowed to co-habit for 3 weeks. The results (Fig. 3) showed that no pregnancies were observed in those groups of mice that received the peptide vaccines based on either $LHRH^{1-10}$ or $LHRH^{6-10}$. In contrast litters were produced by mice inoculated with peptide vaccine based on $LHRH^{7-10}$.

3.3. Peptide vaccines based on $LHRH^{6-10}$ boost the antibody responses elicited by peptide vaccines based on $LHRH^{1-10}$

In order to determine whether or not inoculation with a vaccine based on $LHRH^{1-10}$ predisposes the immune system to respond with antibody direeted to the N-terminal region of the hormone, we determined whether or not a peptide vaceine based on $LHRH^{6-10}$ was able to boost the immune responses elicited by a peptide vaccine incorporating $LHRH^{1-10}$.

Two peptide construets, $T_{H\,morb}$-$LHRH^{1-10}$ and $T_{H\,morb}$-$LHRH^{6-10}$, were used. Two groups of 10 BALB/c mice were inoculated with either $T_{H\,morb}$-$LHRH^{1-10}$ or $T_{H\,morb}$-$LHRH^{6-10}$. Five of the mice that had received $T_{H\,morb}$-$LHRH^{1-10}$ then received $T_{H\,morb}$-$LHRH^{1-10}$ and the other five received $T_{H\,morb}$-$LHRH^{6-10}$ as the second dose of vaceine. Those mice that had received $T_{H\,morb}$-$LHRH^{6-10}$ as the first dose were divided into two groups of five and were either inoculated with $T_{H\,morb}$-$LHRH^{6-10}$ or $T_{H\,morb}$-$LHRH^{1-10}$. All inocula were administered in complete Freund's adjuvant for the first dose and incomplete Freund's adjuvant for the second dose.

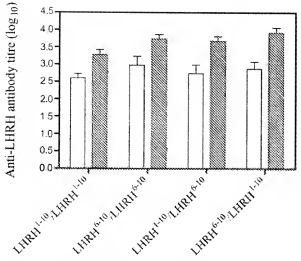

Fig. 4. Ability of peptide vaccines incorporating LHRH$^{6-10}$ to boost the antibody responses elicited by peptide vaccine incorporating LHRH$^{1-10}$. Groups of five mice received a dose of either $T_{H\,morb}$-LHRH$^{1-10}$ or $T_{H\,morb}$-LHRH$^{6-10}$ in complete Freund's adjuvant. On day 28 these mice were boosted with either $T_{H\,morb}$-LHRH$^{1-10}$ or $T_{H\,morb}$-LHRH$^{6-10}$ in incomplete Freund's adjuvant. Blood were taken from animals on days 28 and 42, sera prepared and the antibody titres determined in an ELISA with LHRH$^{1-10}$ as coating antigen. The mean titre obtained from groups of five mice are shown with an error bar representing ±1 S.D. The open bars represent antibody titres obtained in the primary response and the filled bars represent the antibody titres obtained in the secondary response.

The results (Fig. 4) show that there was little or no difference ($p > 0.05$) in the anti-LHRH antibody titres obtained whether or not animals were inoculated with full length LHRH followed by full length LHRH or N-terminally truncated LHRH (LHRH$^{6-10}$). Similarly the anti-LHRH antibody responses elicited by two doses of $T_{H\,morb}$-LHRH$^{6-10}$ were similar ($p > 0.05$) to those obtained when mice received one dose of $T_{H\,morb}$-LHRH$^{6-10}$ followed by one dose of $T_{H\,morb}$-LHRH$^{1-10}$.

The results suggest that a dominant B cell epitope of LHRH is contained within the LHRH$^{6-10}$ sequence.

3.4. Antigenicity of LHRH$^{6-10}$

LHRH$^{1-10}$ or LHRH$^{1-10}$-based conjugate has normally been used as coating antigen in ELISAs to evaluate the immune response of LHRH-based vaccines. The results above suggest that LHRH$^{1-10}$ and LHRH$^{6-10}$ share a dominant B cell epitope. It was of interest therefore to determine whether LHRH$^{6-10}$ could be used as a coating antigen for the ELISAs and whether the antibody titre detected would then correlate with the biological efficacy.

LHRH$^{1-10}$ or LHRH$^{6-10}$ were used to coat plates at a concentration of 5 μg/ml. Anti-LHRH antibody titres in the mouse sera obtained following inoculation with the peptide vaccines containing either LHRH$^{1-10}$ or LHRH$^{6-10}$ with T_{HFlu} as T helper cell epitope were then determined. The results (Fig. 5) show that when LHRH$^{6-10}$ was used as coating antigen, antibody titres detected were significantly lower than those obtained when LHRH$^{1-10}$ was used as the coating antigen. This may be due to

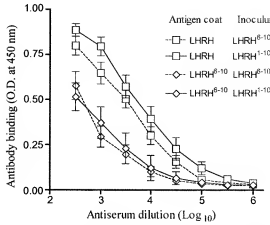

Fig. 5. Comparison of the antigenicity of LHRH$^{1-10}$ and LHRH$^{6-10}$. Either LHRH$^{1-10}$ or LHRH$^{6-10}$ were used to coat plates at a concentration of 5 μg/ml and anti-LHRH antibody titres in sera obtained from mice inoculated with peptide vaccines incorporating LHRH$^{1-10}$ or LHRH$^{6-10}$ and T_{HFlu} determined by ELISA. Mice were inoculated with peptide vaccines on days 0 and 28 and sera were prepared from blood obtained on days 28 and 42.

the fact that LHRH$^{6-10}$ does not coat plates as well as LHRH$^{1-10}$. The low titres obtained therefore make it difficult to differentiate high-responders from low-responders.

In order to overcome the potential problem of weak coating of LHRH$^{6-10}$ we adapted the method described previously (Geysen et al., 1984; Weiner et al., 1992) in which streptavidin is used to coat ELISA plates prior to addition of a biotinylated antigen. In this case the plates were coated with streptavidin before addition of biotinylated LHRH$^{6-10}$. The ability of streptavidin to bind well to plastic and the very high affinity binding of biotin to streptavidin therefore greatly increases the coating of biotinylated LHRH$^{6-10}$ to the plates. As a comparison we also coated the plates with either biotinylated LHRH$^{1-10}$ or LHRH$^{6-10}$ alone. Anti-LHRH antisera obtained from mice before they had been recruited into a fertility trial were used. These mice had received two doses of a lipidated peptide incorporating LHRH$^{1-10}$ as the B cell epitope and T_{HFlu} (Zeng et al., 2002). In this case the lipid moiety Pam2Cys was attached directly at the N-terminus of the peptide and lacks two serine residues. We have shown previously that this *linear* lipopeptide (Fig. 1) is inferior at eliciting anti-LHRH antibody responses when compared with the branched lipopeptide (Fig. 1) (Zeng et al., 2002). Furthermore three mice out of five that had received this linear lipopeptide became pregnant during the fertility trial indicating a less than optimal antibody response in these animals.

The results (Fig. 6) show that when biotinylated LHRH$^{6-10}$ alone was used to coat plates the sensitivity was low or non-existent compared to when biotinylated LHRH$^{1-10}$ was used. Using streptavidin to capture the biotinylated peptide improved greatly the sensitivity of the ELISAs especially when LHRH$^{6-10}$ was used. More importantly, by using streptavidin/biotinylated LHRH$^{6-10}$ we were able to differentiate the anti-LHRH antibody titres of mice according to their pregnancy status; the three mice which subsequently became pregnant demonstrated lower levels of anti-LHRH antibody using streptavidin-coated plates with either biotinylated LHRH$^{1-10}$ or biotinylated LHRH$^{6-10}$.

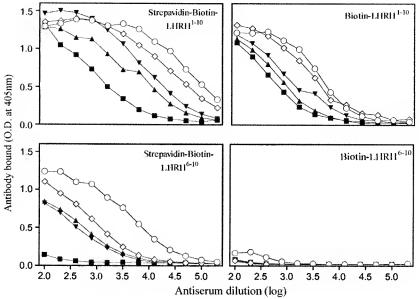

Fig. 6. Comparison of the antigenicity of biotinylated $LHRH^{1-10}$ and biotinylated $LHRH^{6-10}$. $LHRH^{1-10}$ and $LHRH^{6-10}$ biotinylated at their N-termini with a 6-aminohexanoic acid residue spacer, were added to ELISA plates previously coated with streptavidin. Mouse sera obtained on day 42 from mice which had received two doses (days 0 and 28) of linear lipidated peptide vaccines, were examined for anti-LHRH antibody on the various LHRH antigens as indicated in the individual panels. Those of mice which subsequently became pregnant are shown with solid symbols and the mice which were infertile are represented by the open symbols.

4. Discussion

In this paper we have carried out a detailed study to determine whether or not there are any short dominant B cell epitopes within the LHRH hormone sequence. We found that an effective minimal B cell epitope is the C-terminal five-residue pentapeptide $LHRH^{6-10}$. This pentapeptide not only efficiently inhibits the binding of $LHRH^{1-10}$ to anti-$LHRH^{1-10}$ antisera but also elicits a strong anti-LHRH antibody response in its own right when incorporated into a totally synthetic peptide vaccine. Furthermore the mice that had received this peptide were rendered infertile. The other five pentapeptides investigated: $LHRH^{1-5}$, $LHRH^{2-6}$, $LHRH^{3-7}$, $LHRH^{4-8}$ and $LHRH^{5-9}$ showed little inhibitory activity towards the binding of anti-$LHRH^{1-10}$ antibody to $LHRH^{1-10}$. It should be noted that unlike $LHRH^{6-10}$ none of these included the C-terminal glycine indicating that this residue plays an important role in the antigenic properties of LHRH.

The C-terminal tetrapeptide $LHRH^{7-10}$ demonstrated a modest inhibition of the binding of anti-$LHRH^{1-10}$ antibody to $LHRH^{1-10}$. When $LHRH^{7-10}$ was incorporated into a synthetic vaccine it elicited a weak anti-LHRH antibody response following single dose but the response was boosted significantly by a second dose of vaccine. However, the anti-LHRH antibody titres induced by $LHRH^{7-10}$ were significantly lower ($p < 0.05$) than those obtained when either $LHRH^{1-10}$ or $LHRH^{6-10}$ was used and the mice that received the tetrapeptide-based vaccine were not completely protected from pregnancy. These results again indicate that the C-terminal glycine plays an important role in the immunogenicity of LHRH.

The peptide vaccine incorporating $LHRH^{6-10}$ was able to boost the primary anti-LHRH responses elicited by the vaccine incorporating $LHRH^{1-10}$ suggesting that the primary immune response elicited by $LHRH^{1-10}$ is directed to the C-terminal region of this hormone. The peptide vaccine based on $LHRH^{1-10}$ was also able to boost the immune responses elicited by a peptide vaccine incorporating $LHRH^{6-10}$. Taken together we conclude that $LHRH^{6-10}$ is a strong B cell epitope within the LHRH sequence. Although not shown here, our previous studies (Zeng et al., 2005) have demonstrated that long-lived antibody responses are achieved with totally synthetic LHRH-based vaccines encouraging us to the view that antibodies elicited by a vaccine based on $LHRH^{6-10}$ would also demonstrate longevity.

In an attempt to determine if the apparently immunogenically and antigenically dominant epitope $LHRH^{6-10}$ can be used to further analyse the immune response to the hormone we determined whether $LHRH^{6-10}$ could be used as a coating antigen in ELISA-based assay. Our results showed that when $LHRH^{6-10}$ alone was used to coat ELISA plates the antibody titre detectable was very low or non-existent. In order to increase the binding of $LHRH^{6-10}$ we adapted the method from Geysen's group (Geysen et al., 1984; Weiner et al., 1992) by first coating the wells with streptavidin before adding biotinylated $LHRH^{6-10}$. The strong affinity of binding of biotin to streptavidin ensured that adequate amounts of $LHRH^{6-10}$ peptide bound to the well. Much stronger absorbances were observed when $LHRH^{6-10}$/streptavidin was used compared to the absorbances obtained with $LHRH^{6-10}$ (Fig. 5) or biotinylated $LHRH^{6-10}$ (Fig. 6). Furthermore an examination of the titration curves demonstrates that a distinction can be made between those mice that became pregnant and those that did not. It is also possible that the larger difference in the antibody titre between the two non-pregnant mice, which is apparent when biotinylated $LHRH^{6-10}$ is used could reflect a

quantitative difference in their relative levels of fecundity and hormone levels. This could, perhaps be put to use in those situations where discrepancy is sometimes observed between high anti-LHRH antibody titres, testes size and hormone (Turkstra et al., 2001).

LHRH holds enormous potential in the design of an immunocontraceptive vaccines as an alternative to surgical castration which causes great stress to the animals. The problems associated with the use of protein carriers (Ferro et al., 2002a,b; Ferro and Stimson, 1998; Relyveld et al., 1998) can be overcome by use of synthetic T helper cell epitopes such as those used here (Ghosh et al., 2001). The results of the current study indicate not only that synthetic T helper cell epitopes be effectively used but also that short sequences of LHRH are able to elicit strong and biologically effective immune responses An advantage of using short sequences of LHRH is the reduced cost of goods in manufacturing the vaccine. An added advantage is that short homologs may also be used to discern the biological effectiveness of circulating anti-LHRH antibodies.

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